

## The experimental use of antisense oligonucleotides: a guide for the perplexed

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Antisense oligonucleotide biotechnology, at least in theory, provides a specific, rapid, and potentially high-throughput method for inhibiting gene expression and exploring gene function. Over the past decade, a plethora of experiments has been submitted, and a lesser number published, employing this approach to inhibiting gene expression at the mRNA level. The specificity of the antisense approach stems, of course, from the specificity (or, the "information content") of the Watson-Crick base pair interaction.

The fact that antisense oligonucleotides carry significant information in addition to that denoted by their nucleotide sequences is frequently overlooked. This oversight has frequently led to the lack of rigorous controls for many antisense experiments, and, on all too many occasions, to the inappropriate interpretation of experimental data (1). Indeed, serious questions have arisen as to whether an observed biological effect in an antisense experiment has indeed been produced by an antisense mechanism, or whether it is due to a complex combination of non-sequence specific effects. Investigators must therefore understand how to employ antisense technology properly and should recognize its limitations.

### Limitations on sequence specificity

These issues are particularly critical with phosphorothioate oligonucleotides, the class of oligonucleotides most commonly used for antisense applications *in vitro* and *in vivo*. For several reasons, absolute sequence specificity is not attainable using oligonucleotides with phosphorothioate linkages.

Phosphorothioates are used because of their stability in cells and tissues. The substitution of sulfur for a non-bridging oxygen (at each phosphorus in the oligonucleotide chain) is conservative, retaining the charge and solubility of the isosequential phosphodiester oligomer, as well as its ability to hybridize with target mRNAs (2). Nevertheless, the result of this substitution is a very different, and very biologically active, chemical entity. Phosphodiester and phosphorothioates are polyanions, and as such, they are capable of binding to proteins that contain polyanion binding sites. Such proteins include, but are not limited to, a large number of heparin bind-

ing proteins (3, 4), such as bFGF, PDGF, VEGF, EGF-R (5), CD4, gp120 (6), Mac-1 (7), laminin, fibronectin, and many others (8). The affinity of phosphorothioate oligonucleotides for such proteins is length-dependent but largely sequence-independent (9). This affinity can be in the low nanomolar range, and phosphorothioate oligonucleotides of the appropriate length can block the binding of heparin-binding proteins to their natural receptors. Since these effects are biologically relevant, it therefore is important to optimize oligonucleotide length, and even more importantly, to minimize oligonucleotide concentration.

Sequence-nonspecific effects of longer phosphorothioate — and indeed phosphodiester — oligonucleotides also arise because of the limited requirement of ribonuclease (RNase) H for long double-stranded substrates. RNase H, a ubiquitous enzyme that recognizes the mRNA strand of an RNA-DNA duplex (10), cleaves the target mRNA to provide the major mechanism of the antisense effect. This enzyme is highly specific with regard to its substrate's backbone: Only charged oligodeoxyribonucleotide phosphodiester and phosphorothioates elicit efficient RNase H activity (11), whereas noncharged oligonucleotides, including peptide nucleic acids, morpholino-oligos, and 2'-O-alkyloligoribonucleotides do not. However, RNase H does not require full-length homology between the target mRNA and the incoming antisense oligonucleotide to recognize and cleave an mRNA/DNA duplex (12). In fact, at least in cell-free systems, RNase H can recognize a duplex as small as a 6mer. While the duplex length required may be somewhat greater in living cells (13), the ability of the enzyme to cleave short duplexes results in a seemingly paradoxical drop in target specificity as the length of an antisense oligonucleotide increases. This drop occurs because of the greater number of short sequences, nested within a large oligonucleotide, that can be targeted by RNase H. For this reason, too, the length of an antisense oligonucleotide must be optimized: If the antisense oligonucleotide is either too long or too short, an element of specificity is lost. At the present time, the optimal length for an antisense oligonucleotide seems to be roughly 16–20 nucleotides.

### Guidelines for the use of antisense oligonucleotides

The following guidelines apply to the use of any charged oligonucleotide species, and represent, I believe, the present state of the art.

(a) Although computer-based approaches are beginning to appear, it is still necessary to choose the antisense oligonucleotide sequence from a panel of oligonucleotides, e.g., by mRNA "walking."

Consensus opinion, based on years of observation, holds that for every eight antisense oligonucleotides

tested, approximately one is effective in targeting a specific mRNA. In many published papers, of course, the authors do not generate a panel of oligonucleotides, but claim activity after examining only one, or at most two, species. In a sobering analysis, Tu, et al. (14) examined 2,026 published reports of putatively successful antisense inhibition. In 82% (1,655 experiments), only one antisense oligomer was evaluated. In 12.2% (248 experiments) only two or three were tested. Only in 3.9% (81 experiments) were from four to nine evaluated, and in only 2.1% (42 experiments), were more than ten checked. Therefore, about 94% of the experiments were deemed successful, although the vast majority of these used only a single oligonucleotide! These statistics cannot be easily reconciled with the virtually universal experience that only approximately one in eight (12.5%) of the putative antisense oligonucleotides tested can be shown to be active. Some of the explanation may lie with publication biases, since oligomers with no biological activity are unlikely to be reported. However, in many, and perhaps most of the citations in which only a single oligomer was evaluated, the results reported may represent some combination of true antisense effects with sequence-nonspecific and cytotoxic effects, all included, unsorted, under the "antisense" moniker.

Therefore, all antisense oligonucleotides must be generated from a panel of putative candidates (see Dean et al., ref. 15; Monia et al., ref. 16; and Lebedeva et al., ref. 17). The panel may consist of all-phosphorothioate oligonucleotides, which are available from commercial sources and are relatively inexpensive. For improved specificity (due to decreased RNase H cleavage of non-targeted mRNAs), additional experiments may subsequently be performed with isosequential "gap-mers," which contain chimeric phosphorothioate backbones, consisting of oligoribonucleotides at each end and oligodeoxyribonucleotides in the middle. The "controls" are therefore the large numbers of putative antisense oligonucleotide candidates that do not prove to be active in pilot experiments. There is no evidence that "scrambled" oligonucleotides provide superior controls. Additional single- or double-based mismatched controls are useful and confirmatory, but otherwise not required. The demonstration of dose-dependent downregulation is also important and convincing. Other useful controls might include (i) the use of isosequential oligonucleotides with two different backbones. If the inhibition of a molecular target (see b below) is identical for both, the case for an antisense mechanism is strengthened. (ii) the use of two or more oligonucleotides of different sequences that are complementary to the same target. If the observed phenotypes are similar and are distinct from those seen using control oligonucleotides, an antisense mechanism of target downregulation would be strongly suggested. (iii) introduction of the target gene with one or more mutations in the region complementary to the antisense oligonucleotide. Lack of antisense inhibition in this case is suggestive, particularly if the antisense oligomer is still effective when the wild-type target is forcibly overexpressed. (iv) in addition to the use of antisense oligonucleotides, the inhibition of expression

of a molecular target by antisense RNA strengthens the case for an antisense mechanism.

(b) Downregulation of a relevant molecular target (usually protein expression) must be demonstrated.

Except under rare and strongly justified circumstances, the use of an observed biological endpoint to claim antisense efficacy is not acceptable. Because antisense oligonucleotides, particularly those with phosphorothioate backbones, are sequence-nonspecifically active, it is logically circular to claim that an observed biological effect is due to specific antisense effects. Instead, the "gold-standard" of antisense efficacy is downregulation of a molecular target, most often protein expression (usually as demonstrated by Western blotting), with or without downregulation of mRNA expression. If protein expression is diminished while mRNA expression is unaffected, the possibility of non-sequence specificity should be considered, especially if a charged oligonucleotide has been employed.

Naturally, such pilot experiments cannot address the possibility that the oligonucleotides chosen have non-sequence specific activities, so other controls are needed for reassurance on this point. Furthermore, when phosphorothioates are employed, it is generally not possible to deduce that an observed biological effect, even in the presence of presumed antisense downregulation of the targeted protein, is caused by the downregulation of that target. Indeed, the only claim that is usually justified from such experiments is that the downregulation of the target may be necessary for the observed biological effect. Evidence that it is not only necessary, but also sufficient is usually not available from these studies. This critical distinction has almost invariably been overlooked.

(c) Unprotected all-phosphodiester oligonucleotides should not be used in antisense experiments.

Not only are these molecules nuclease-sensitive, but their nucleotide monophosphate degradation products (in particular dGMP) may be toxic, perhaps in part due to their ability to inhibit ribonucleotide reductase (18). Individual phosphodiester linkages may be employed in an antisense oligonucleotide, particularly if it is protected at the 3' and 5' termini with, for example, three phosphorothioate linkages. It should also be pointed out that phosphodiester linkages 5' to a purine residue are almost as nuclease resistant as a phosphorothioate — it is the linkages that are 5' to a pyrimidine that are highly nuclease-sensitive. Therefore, the use of chimeric phosphorothioate/phosphodiester backbones is acceptable in an antisense experiment.

(d) Maximize sequence specificity and minimize sequence nonspecificity.

The antisense oligonucleotide concentration, particularly if phosphorothioates are employed, must be kept to a minimum to avoid sequence nonspecific effects and the ectopic cleavage of mRNAs by RNase H. Hence, except under unusual and well-justified circumstances, oligonucleotides (especially phosphorothioates) should not be delivered "naked" (i.e., without a carrier) to cells

in tissue culture — a concern that does not apply to experiments performed in experimental animals. Very high concentrations (usually  $\geq 20 \mu\text{M}$ ) of naked oligonucleotides are required for “antisense” efficacy when no carrier is used. At such concentrations, tremendous non-sequence specificity will be produced, at least in part because of the adsorption of the oligonucleotide to cell-surface heparin-binding proteins. Therefore, the delivery of naked oligonucleotides should be avoided, and putative evidence for antisense effects obtained in this manner should be deemed highly suspect. Oligonucleotides (19) may be delivered instead using any one of a number of commercially available carriers, including the cationic lipids Lipofectin, Lipofectamine 2000 (both, Life Technologies Inc., Grand Island, New York, USA), and Cytofectin (Glen Research, Herndon, Virginia, USA), as well as polyamines.

The ratio of carrier to oligonucleotide should be optimized. It is advisable to use the lowest effective concentration of carrier, since it is possible that carriers are toxic or could produce as yet unknown effects on cells, confounding evaluation of gene function. Also note that the proper control for a carrier is not treatment of targeted cells with carrier alone; rather, it is treatment of cells with carrier complexed to “control” oligonucleotides. Nevertheless, even when employing a carrier, except under unusual and well-justified circumstances, it is best to avoid phosphorothioate oligonucleotide concentrations of greater than 4–5  $\mu\text{M}$ .

(e) Do not employ antisense oligonucleotides containing four contiguous guanosine residues.

Oligonucleotides of this type can form G-quartets and tetraplexes via Hoogsteen base-pair formation (20). This ability (which depends on oligomer sequence and even more so on the relative position of the guanosines in the oligomer) may not only decrease the concentration of available single-stranded oligomer, but also may create new, highly charged structures that may have numerous sequence-nonspecific effects. This problem can be overcome if one of the guanosine residues is substituted by 7-deazaguanosine, which cannot form Hoogsteen base pairs (21).

(f) In animal experiments, do not use oligonucleotides that contain an unmodified CpG motif.

Phosphorothioate oligonucleotides stimulate immune responses, a property that is vastly enhanced by the presence of the CpG motif (22). CpG-dependent immune stimulation is Th1-biased, but even SCID mice (23) can demonstrate increased levels of IFN- $\gamma$  and IL-12 following treatment with these agents (24). Reports of antisense-based antitumor effects in nude or SCID mice bearing human tumor xenografts that have not controlled for the CpG motif are therefore suspect, and most likely represent cross-species tumor rejection due to nonspecific immune stimulation. Furthermore, the data suggest that the sequences flanking the CpG motif strongly influence the intensity of the immunostimulation, thus making “scrambled” oligonucleotide controls essentially irrelevant. C5-methylation or alkylation

of the cytosine residue appear to abolish most of the CpG-associated immune stimulation and offer one way of controlling this problem (22).

As with CpG sequences, palindromic sequences can be immune-stimulating and should also be avoided for antisense applications in vivo.

### The future of the antisense approach

As with other reagents, antisense oligonucleotides can be used appropriately only by adhering to an increasingly well-defined set of rules. These rules will certainly evolve as novel classes of antisense oligonucleotides and related molecules are introduced to the community. Indeed, it is possible to foresee a situation in which oligonucleotide specificity has increased to the point where many of the rules discussed here have become moot. However, until such a time, I remain convinced that an appropriately flexible adherence to the above guidelines, coupled with further mechanistic research, will allow the vast potential of this technology to be fully realized.

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